

# Intraphagolysosomal pH in Canine and Rat Alveolar Macrophages: Flow Cytometric Measurements

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Intracellular dissolution of inhaled inorganic particles is an important clearance mechanism of the lung and occurs in phagolysosomal vacuoles of phagocytes. Flow cytometric measurements of intraphagolysosomal pH in alveolar macrophages (AM) obtained from beagle dogs, Wistar rats, and from a baboon were made using fluorescein isothiocyanate-labeled amorphous silica particles (FSP). AM were obtained by bronchoalveolar lavage. FSP were phagocytized by AM in cell suspensions incubated in full media for 24 hr up to 6 days. Dual laser flow cytometry was performed and six-parameter list mode data were recorded from forward scatter, side scatter, and fluorescence intensities at 530 nm excited at 457 nm and 488 nm as well as logarithmic fluorescence intensity at wavelengths 630 nm excited at 488 nm. In this way it was possible to discriminate viable AM with phagocytized FSP from lysing AM with phagocytized FSP and from cells without FSP and from free FSP. Viable cells were distinguished from lysing cells by staining with propidium iodide immediately before the flow cytometric measurement. A calibration curve for the pH value was determined from FSP suspended in buffered media at pH values ranging from 3.5 to 7.5. First flow cytometrical results indicated that after an incubation time of 24 hr, the mean intraphagolysosomal pH of viable AM was  $4.7 \pm 0.3$  for dogs and  $5.1 \pm 0.5$  for rats. The intraphagolysosomal pH of the baboon AM was 4.5.

## Introduction

Alveolar macrophages (AM) constitute an important defense mechanism of the lungs against inhaled aerosol particles (*1*). It is well established that particles deposited in the alveolar region that are not readily soluble will be phagocytized by AM. Intracellular dissolution of ingested particles is one important function of AM and is the first step of the clearance process of translocation of inhaled material from the lungs to blood (*2-7*). In fact, cultured AM of various species were shown to dissolve various test particles at a faster rate than culture medium (*2-6,8,9*). It was shown that the pH of the phagolysosomal plasma at about 5 is lower than that of extracellular body fluids (*10-14*). Therefore, the intraphagolysosomal pH (PL-pH) is believed to

be an important parameter of the intracellular particle dissolution, which is the initial step of an essential clearance mechanism: the translocation to blood.

Fluorescein isothiocyanate (FITC)-labeled organic or inorganic particles were used as a probe to measure the pH within the phagolysosomal vacuole because the emitted fluorescence intensity of fluorescein is pH dependent (*10-16*). The intensities of the emitted fluorescent light at two excitation wavelengths were measured to calculate their ratio. This parameter is a nonlinear, monotonic function of pH and is independent of the amount of FITC and the size of the particles as well as the number of phagocytized particles (*10,11,15,16*). The measured pH value did not change when the number of FITC-labeled amorphous silica particles (FSP) added to rabbit AM cultures was varied within a factor of 10 (*13*). Measurements of phagolysosomal pH either in individual AM based on a microscopic cytofluorometric technique or in a population of AM using a fluorescence spectrophotometer showed the same results (*12*). FSP are more satisfactory for this purpose than organic particles because they are more inert and less soluble.

Recently (*13*), mean pH values for rabbit AM were compared when FSP were phagocytized in culture (*in vitro*) and *in vivo* after intratracheal instillation and retention for 24 hr and 1 week. The

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pH measured *in vitro* ( $5.1 \pm 0.1$ ) was significantly higher than the pH measured *in vivo* at 24 hr ( $4.9 \pm 0.1$ ) and at 1 week ( $4.5 \pm 0.1$ ) after instillation. In spite of the rather small variation of the pH in AM obtained from one rabbit, significant differences existed among rabbits (12). Measurements of AM obtained from bronchoalveolar lavage (BAL) of baboons, dogs, guinea pigs, and rabbits resulted in an average pH of 4.8 or 4.9 with small variations among and within animals (14).

The objectives of the present study were *a*) to develop a time-saving method to measure the PL-pH in AM based on a flow-cytometric technique using FSP as a probe, *b*) to classify simultaneously both viable and lysing AM with phagocytized FSP by staining with propidium iodide, *c*) to monitor the influence of different FSP: cell ratios and the effects of different media, and *d*) to compare the mean PL-pH of canine and rat AM.

## Materials and Methods

### Animals and Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was carried out on 14 untreated, clinically healthy, 2 ( $\pm 1$ )-year-old beagle dogs of both sexes and on 12 male, about 2-month-old, specific pathogen-free, inbred Wistar rats (body weight  $250 \pm 10$  g). Animals of both species were bred at our animal facilities. The dogs were anesthetized using intravenous thiobarbiturate injections (Surital, Parke-Davis; 17.4 mg/kg body weight); additional doses were given as necessary. BALs were performed with a fiberoptic bronchoscope (Pentax, model FB-15H, Asahi Optical Co., Tokyo, Japan). Under direct visualization, the bronchoscope was led carefully into a subsegmental bronchus and then a balloon at the tip of the bronchoscope was inflated. Three aliquots of 15 mL sterile phosphate-buffered saline (PBS, PM 16 buffer, Serva, Heidelberg; without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) at body temperature were instilled via the instrument channel and gently aspirated into a suction trap after 10 sec. A total of 135 mL of saline was instilled per dog, divided equally among subsegmental bronchi in three different lobes of the right lung. The volume of the recovered BAL fluid was 60–70% of the instilled volume.

The rats were anesthetized by intraperitoneal barbiturate injections (Nembutal, 50 mg/kg body weight) and exsanguinated. Subsequently, the lungs were excised and the trachea cannulated. Aliquots of 5 mL sterile PBS at body temperature were rinsed through the cannula into the total lung. Lungs were gently massaged while inflated and then the BAL fluid was carefully aspirated into a syringe. After administration of a total of  $6 \times 5$  mL sterile PBS, about 27 mL of BAL fluid were recovered.

The processing of the BAL fluid was the same for both animal species. Immediately after lavage, the BAL fluid was filtered through sterile gauze and centrifuged for 15 min at 400g. The cell pellet was resuspended in PBS and total cell counts were made using a hemocytometer. The viability of the cells was determined by trypan blue exclusion. A differential count of 600 BAL cells was performed on Pappenheim-stained cytocentrifuge preparations (Cytospin 2, Shandon Southern Instruments) using standard morphologic criteria (17).

Cells lavaged from baboon lung were obtained from J. -L. Poncy (Commissariat à l'Energie Atomique Bruyère-le-Châtel, France) and transported to Munich within 7 hr. After an incubation time of 24 hr, the PL-pH was measured.

### BAL Cell Incubation with FSP

FSP were obtained from the Karolinska Institute, Stockholm, Sweden. The preparation of FSP (diameter  $3.0 \mu\text{m}$ ) has been described previously (12,13). Cells were incubated in complete medium (pH 7.4, RPMI-1640 without phenol red, Serva, Heidelberg; supplemented with 5% fetal calf serum, 100 units penicillin, and 100  $\mu\text{g}$  streptomycin/mL, Gibco) in 50-mL polypropylene centrifuge tubes and kept in an incubator at  $37^\circ\text{C}$ , <95% relative humidity, and 5%  $\text{CO}_2$  in air. An equal number of FSP were added to  $1 \times 10^6$  BAL cell culture medium. Before sampling for the pH test, the tubes were gently vortexed to resuspend the cells. Because  $1\text{--}2 \times 10^5$  cells were needed for a single flow-cytometric measurement, the pH of AM in the same preparation could be measured after various incubation times with FSP varied from 24 hr to 6 days. For all cell measurements,  $10^4$  or more events were accumulated by the flow cytometer.

In a few additional experiments the FSP: cell ratio was changed from 0.5:1 to 5:1, and different cell culture media were used RPMI without phenol red, M-199 with phenol red and PBS to study their influence on the flow cytometric measurements.

### Flow Cytometry

Dual laser flow cytometry was performed on a FACStarPLUS flow system (Becton Dickinson, Mountain View, CA) equipped with two argon-ion lasers and a nozzle tip ( $70 \mu\text{m}$  diameter) for jet in air sample interrogation. The excitation wavelengths were set to 457 nm (250 mW) and 488 nm (500 mW). Six parameters were recorded as shown in Table 1.

The FLA488/530 and FLA457/530 fluorescence parameters were used to calculate the ratio for determination of the pH value of the FSP (Fig. 1): FSCHL and SSCHL were used to characterize the AM population. FLA488/530L was used to give a better separation of cells with and without phagocytized FSP and to control the performance of the system (Fig. 1). The FLA488/630L fluorescence parameter registered the propidium iodide staining of cells to determine cells with an intact membrane (later defined as viable cells versus cells with a permeable membrane (later defined as "dead cells") (Fig. 1).

The data analysis system (DAS V3.21) recently developed in the Institut für Biophysikalische Strahlenforschung was used. For all cell measurements, the free FSP were gated initially in the (SSCHL)-(FLA488/530L) parameter plane (Fig. 1). The second gate was set around the high propidium iodide stained fraction ("dead cells") and the third one around the low propidium iodide stained fraction ("viable cells"). The fourth population with intermediate (FLA488/630L) fluorescence was gated separately

Table 1. Parameters recorded for flow cytometry.

| Parameter   | Description                  | Scale and wavelength                                  |
|-------------|------------------------------|---|
| FSCHL       | Forward scatter pulse height | Logarithmic scale                                     |
| SSCHL       | Side scatter pulse height    | Logarithmic scale                                     |
| FLA488/530  | Fluorescence pulse area      | Excitation 488 nm, emission 530 nm, linear scale      |
| FLA488/630L | Fluorescence pulse area      | Excitation 488 nm, emission 630 nm, logarithmic scale |
| FLA457/530L | Fluorescence pulse area      | Excitation 457 nm, emission 530 nm, linear scale      |

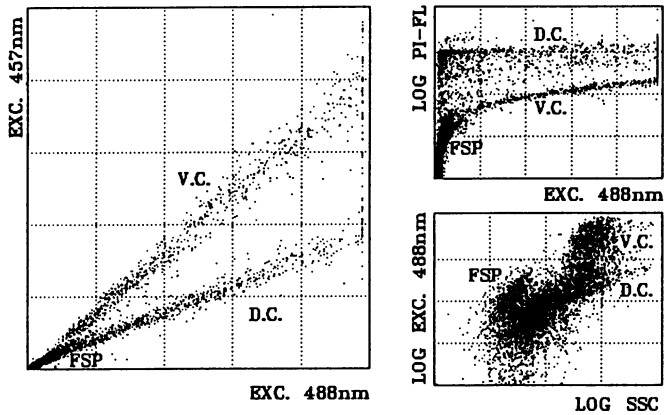


FIGURE 1. Dot plot analysis of a sample of dog alveolar macrophages which had phagocytized fluorescein isothiocyanate-labeled silica particles (FSP). V.C., viable cells; D.C., dead cells; FSP, free FSP. (Left) PHRATIO, (FLA488/530)/(FLA457/530); (upper right) (FLA488/530)-(FLA488/630L); (lower right) (SSCHL)-(FLA488/530L).

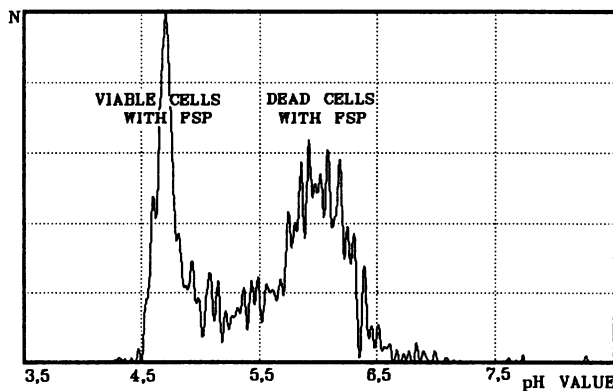


FIGURE 2. Graph of the distribution of the intraphagolysosomal pH in a sample of dog alveolar macrophages.

between the dead and viable cells and was later defined as intermediate or lysing cells. Gates 2–4 were done in the (FLA488/530)-(FLA488/630) parameter plane (Fig. 1).

The quotient (FLA488/530)/(FLA457/530) was evaluated for each event and is referred as PHRATIO (Fig. 1). Mean values of this quotient for the viable cell fraction, as well as for the dead cell fraction, were calculated and transformed to pH values using the calibration curve. To analyze the distribution of the pH in cells with FSP, a one-dimensional histogram was generated (Fig. 2).

## Calibration Curves

Calibration curves were obtained from FSP suspended in full medium mixed with 0.1 N HCl to give buffer solutions in the pH range 3.5–7.5 in steps of 0.5 pH units. The pH of mixtures were adjusted in Eppendorff tubes by thorough vortexing and ultrasonic agitation. Immediately before the flow cytometric measurement, the pH value of the sample was rechecked using

a microprobe pH meter. The calibration curve was determined by calculating PHRATIO for all FSP in the calibration samples and plotting the mean values of the quotients versus the measured pH values (Figs. 3 and 4). For each pH value of the calibration curve, a minimum of 5000 FSP were recorded. Forward scatter (FSCHL) was used as an on-line trigger parameter.

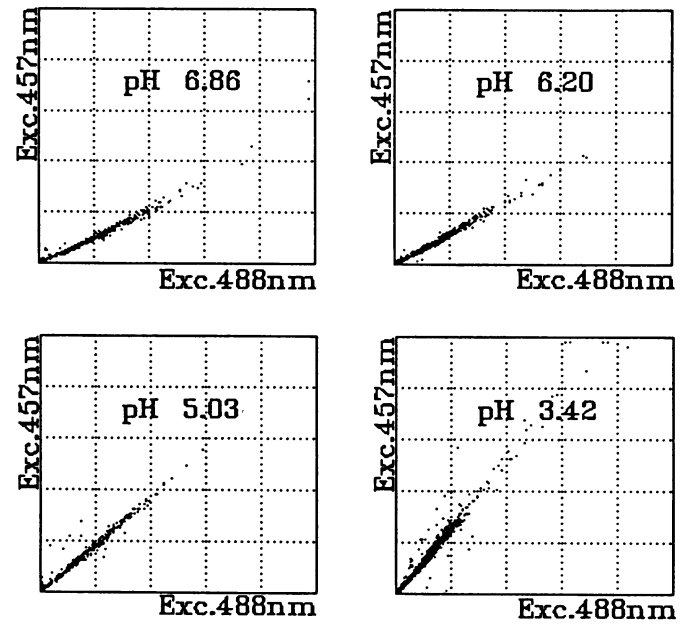


FIGURE 3. Dot plots obtained from flow cytometric measurements to evaluate the pH calibration curve of fluorescein isothiocyanate-labeled silica particles in full medium. Exc. 457 nm, fluorescence area (FLA457/530), Exc. 488 nm, fluorescence area (FLA488/530).

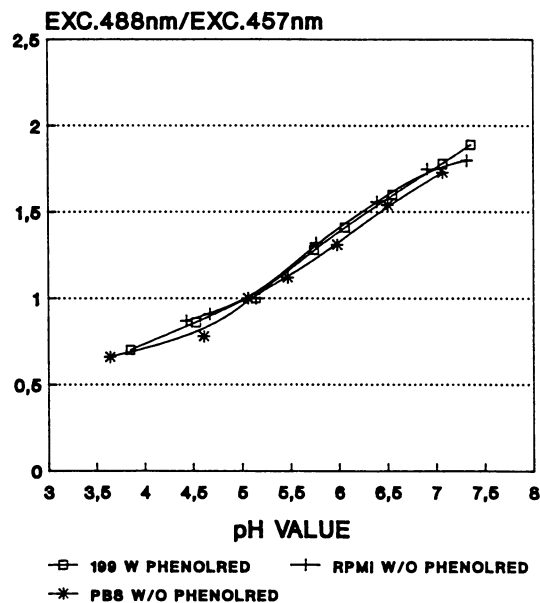


FIGURE 4. Calibration curves of the pH value using fluorescein isothiocyanate-labeled silica particles in different media. Exc. 488 nm/Exc. 457 nm = PHRATIO = FLA488/530)/(FLA457/530). (□) 199 with phenol red; (+) RPMI without phenol red; (\*) PBS without phenol red.

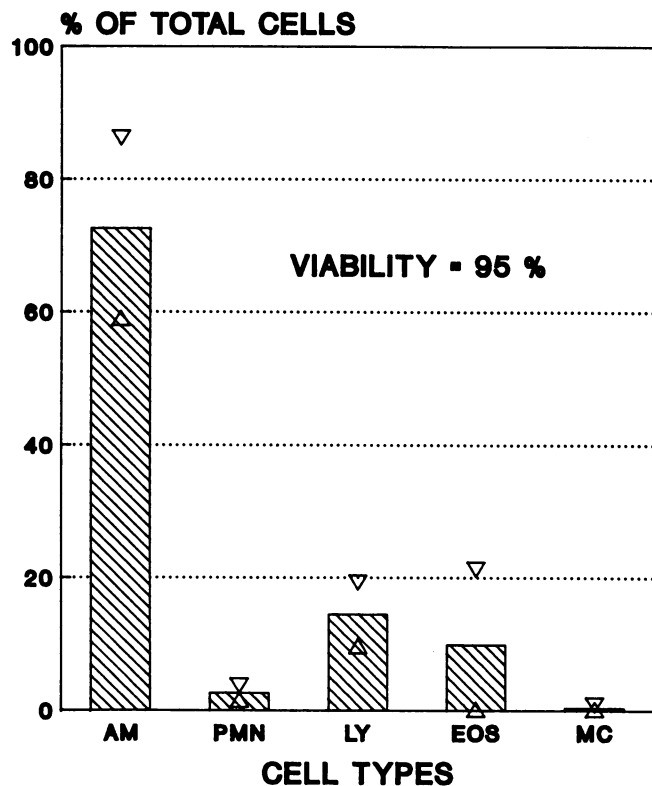


FIGURE 5. Differential counts of bronchoalveolar lavage cells obtained from in beagle dogs ( $n = 14$ ; mean  $\pm$  SD). AM, alveolar macrophages; PMN, polymorphonuclear granulocytes; LY, lymphocytes; EOS, eosinophilic granulocytes; MC, mast cells.

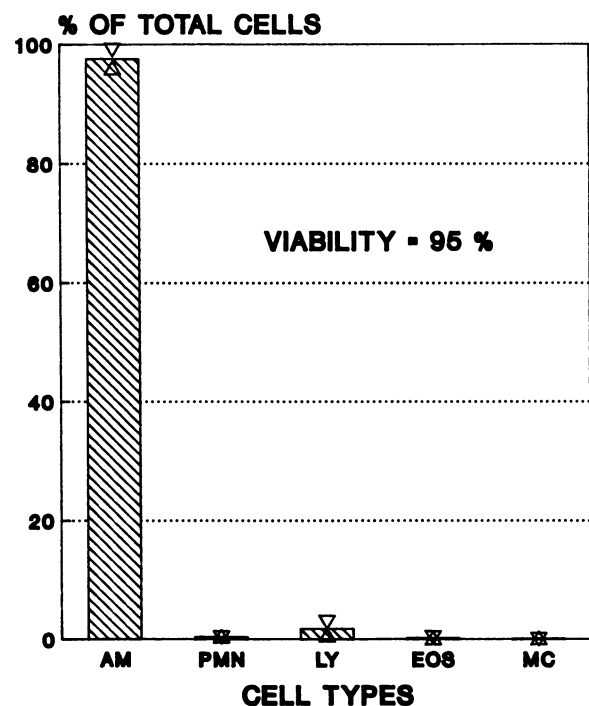


FIGURE 6. Differential counts of bronchoalveolar lavage cells obtained from Wistar rats ( $n = 12$ ; mean  $\pm$  SD). AM, alveolar macrophages; PMN, polymorphonuclear granulocytes; LY, lymphocytes; EOS, eosinophilic granulocytes; MC, mast cells.

## Results and Discussion

### BAL Cells

Differential cell counts of lung lavages from beagle dogs and from SPF Wistar rats yielded predominantly AM with much smaller numbers of lymphocytes and eosinophilic and polymorphonuclear granulocytes (Figs. 5 and 6). The differential cell counts showed the typical BAL cell distribution found in these species. In the BAL fluid of the baboon, we found 87.5% AM, 3.3% polymorphonuclear granulocytes, 7.3% lymphocytes, 1.8% eosinophilic granulocytes, and 0.3% mast cells. This result is in good agreement with differential cell counts of BAL fluid from two baboons from the same source (14).

Aliquots of samples for flow cytometric measurements obtained from each species were also examined by fluorescence microscopy. Predominant fractions of AM were confirmed which did not contain FSP. Other cell types were only seen in the first few hours of incubation.

### Calibration Curves

Before the pH measurement of cell samples, the calibration curve was determined. Starting from pH 3.5, PHRATIO increased rapidly with increasing pH (Fig 3). Usually the standard deviation of the calculated PHRATIO was around 5%. Particularly in the pH range of interest (pH 3.5–5.5), the calibration curves were highly reproducible. There were no significant differences between suspension of FSP on PBHS, RPMI 1640, and M-199, the latter with phenol red (Fig. 4). Nevertheless, all

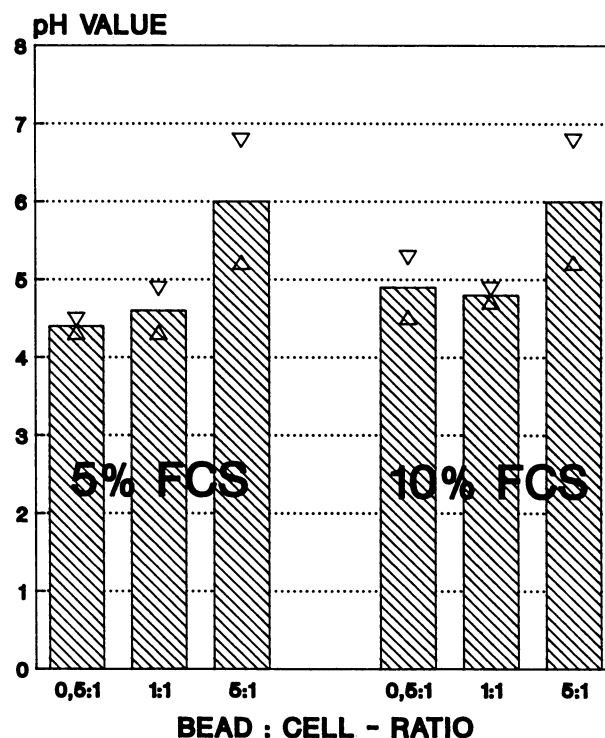


FIGURE 7. Influence of particle:cell ratio and fetal calf serum (FCS) concentration on intraphagolysosomal pH of dog alveolar macrophages ( $n = 4$ ; 24-hr incubation).

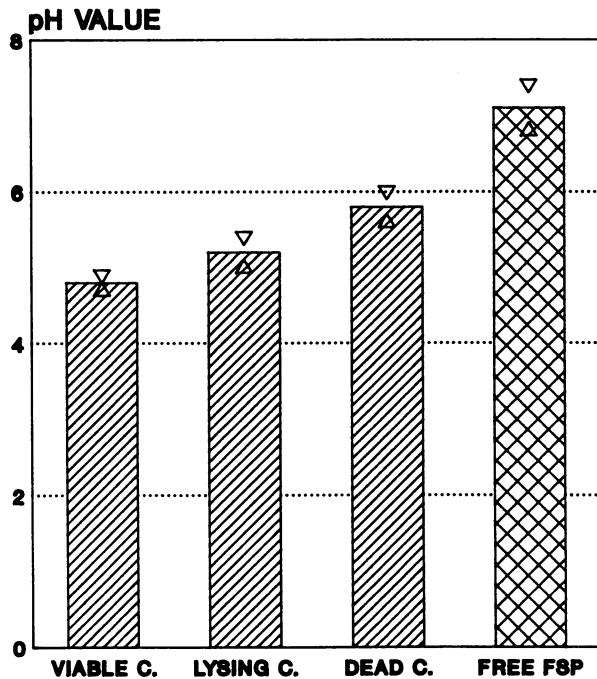


FIGURE 8. Influence of dead cells on measurement of intraphagolysosomal pH of dog alveolar macrophages, 3-day incubation. ( $n = 4$ ). Viable C, viable cells; Lysing C, lysing cells; Dead C, dead cells; FSP, fluorescein isothiocyanate-labeled silica particles.

cell samples obtained from dogs and rats were cultured in full medium using RPMI 1640 without phenol red. The baboon cells were cultured in medium 199 with phenol red. The concentration of 5% or 10% fetal calf serum in the RPMI medium 199 with phenol red. The concentration of 5% or 10% fetal calf serum had no significant effect on the PL-pH of the canine AM (Fig. 7).

### Intraphagolysosomal pH Measurement

A clear distinction between membrane-attached and internalized FSP in AM was not possible. The presence of free FSP in the culture medium indicated the probability of particles attached to cells, i.e., the more free FSP detected in the cell suspension, the more membrane-attached FSP. The attached FSP showed the same pH as the free FSP in the culture medium and caused misleading results of PL-pH (Figs. 7 and 8; bead: cell 5:1). Therefore, it was desirable to have only a small fraction of free FSP. If the particle:cell ratio was 1:1 or lower, free FSP were rarely detected in the culture medium after 24 hr of incubation; as a result the pH varied less and the mean pH was the same for both particle: cell ratios studied (Fig. 7). Adding some FSP immediately before the pH measurement allowed the determination of the pH of the medium. This confirmed the pH of the buffered medium and proved to be an important control.

Because FSP were heterogenous in size and amount of FITC label, the applied flow cytometric method did not allow determination of the number of FSP phagocytized by the cells. In spite of this, a significant change of pH with increasing fluorescence, indicating increasing numbers of phagocytized FSP per cell, was not observed, as shown by the constant slope, which is PHRATIO (Fig. 1). Our studies confirmed the results of Nyberg et al.

(12,13) that the measurement of the PL-pH was not affected by the size of FSP or the amount of FITC.

We used BAL cells without any cell separation procedure, which might have changed cell functions because AM were distinguished from other cells by FSCHL and SSCHL. Because we were only interested in the PL-pH of functional AM, exclusion of lysing or dead cells at the time of measurement was found to improve the method substantially. Using the cell classification based on propidium iodide exclusion, we found a significant increase of PL-pH from viable to lysing and to dead cells (Fig. 8). For this reason the propidium iodide positive intermediate and dead cells were excluded from calculation of PL-pH.

The graph of pH distribution had two clearly separated peaks (Fig. 2). The viable cells showed only a slight variation of PL-pH values in contrast to the fraction of lysing and dead cells. The half width of the distribution in viable AM was usually 0.3 pH units (Fig. 2).

Our data are in good agreement with those found in AM of rabbits (12,13) and with those obtained from an interspecies comparison, including AM of dogs, rabbits, guinea pigs, and baboons, using a fluorescence spectrophotometer (14). The latter study reported an average PL-pH in AM of beagle dogs and baboons of  $4.8 \pm 0.2$  and  $4.8 \pm 0.1$ , respectively. In the present study the PL-pH in AM of rats ( $n = 12$ ) was determined for the first time and found to be  $5.1 \pm 0.5$  using FSP as a probe (incubation time 24 hr). Thus PL-pH values found in rat AM ( $5.1 \pm 0.5$ ) are not significantly different from those found in dog AM ( $4.7 \pm 0.3$ ;  $n = 14$ ). The PL-pH in the baboon AM was 4.5 and was comparable with the values of the interspecies comparison (14).

We did not find significant changes of PL-pH in dog AM during the first 3 days of incubation with FSP. The PL-pH was  $4.7 \pm 0.1$  on the first day and  $4.8 \pm 0.1$  on the third day of incubation ( $n = 4$ ), values that agree well with the results in rabbit AM (13). Nyberg et al. (13) found a decrease of the PL-pH in rabbit AM after intratracheal instillation of FSP and subsequent lung retention for 6 days, as we found in dog AM cultured with FSP for 6 days (PL-pH  $3.9 \pm 0.2$ ;  $n = 4$ ).

Although no consecutive BAL are possible in rats or other rodents, we looked for intrasubject variations of PL-pH in dog AM obtained from three dogs at two different time points (4 weeks apart; 24-hr incubation). The mean value of PL-pH was 4.5 (minimum 4.35; maximum 4.7) in July and 4.7 (minimum = 4.6; maximum = 4.7) in August 1990. Thus, within 4 weeks, the PL-pH of AM varied by not more than 0.3 pH units within the individual and between different dogs. These results are similar to findings in AM of rabbits (12).

### Conclusion

The flow cytometrical measurement of PL-pH in BAL cell suspensions was developed using FSP as a probe. The advantages of the flow cytometrical pH measurement are a) measurement of the PL-pH in a viable AM population through elimination of lysing or dead cells (propidium iodide staining); b) saving time: a sample could be measured in 2–3 min (i.e., 10000 events, respectively, 1000–2000 viable AM with phagocytized FSP); c) high statistical precision due to analysis of 1000–2000 viable cells with FSP; and d) possibility to sort cells of interest for further investigations.

The flow cytometrically determined PL-pH values in canine and in baboon AM were comparable with those determined earlier. Because the intersubject variability of the PL-pH was rather large in all species studied, no significant differences in or between the species were found.

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